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ORIGINAL ARTICLE

A long noncoding RNA critically regulates Bcr-Abl-mediated cellular transformation by acting as a competitive endogenous RNA

G Guo[1], Q Kang[1], X Zhu[1,2], Q Chen[3], X Wang[1], Y Chen[1], J Ouyang[1], L Zhang[4], H Tan[5], R Chen[6], S Huang[7] and J-L Chen[1,3]

Aberrant expression of long noncoding RNAs (lncRNAs) is associated with various human cancers. However, the role of lncRNAs in Bcr-Abl-mediated chronic myeloid leukemia (CML) is unknown. In this study, we performed a comprehensive analysis of lncRNAs in human CML cells using an lncRNA cDNA microarray and identified an lncRNA termed lncRNA-BGL3 that acted as a key regulator of Bcr-Abl-mediated cellular transformation. Notably, we observed that lncRNA-BGL3 was highly induced in response to disruption of Bcr-Abl expression or by inhibiting Bcr-Abl kinase activity in K562 cells and leukemic cells derived from CML patients. Ectopic expression of lncRNA-BGL3 sensitized leukemic cells to undergo apoptosis and inhibited Bcr-Abl-induced tumorigenesis. Furthermore, transgenic (TG) mice expressing lncRNA-BGL3 were generated. We found that TG expression of lncRNA-BGL3 alone in mice was sufficient to impair primary bone marrow transformation by Bcr-Abl. Interestingly, we identified that lncRNA-BGL3 was a target of miR-17, miR-93, miR-20a, miR-20b, miR-106a and miR-106b, microRNAs that repress mRNA of phosphatase and tensin homolog (PTEN). Further experiments demonstrated that lncRNA-BGL3 functioned as a competitive endogenous RNA for binding these microRNAs to cross-regulate PTEN expression. Additionally, our experiments have begun to address the mechanism of how lncRNA-BGL3 is regulated in the leukemic cells and showed that Bcr-Abl repressed lncRNA-BGL3 expression through c-Myc-dependent DNA methylation. Taken together, these results reveal that Bcr-Abl-mediated cellular transformation critically requires silence of tumor-suppressor lncRNA-BGL3 and suggest a potential strategy for the treatment of Bcr-Abl-positive leukemia.

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INTRODUCTION

Chronic myeloid leukemia (CML) is a hematological malignancy associated with a translocation between chromosome 9 and 22 that results in the formation of *bcr-abl* hybrid gene which occurs in over 90% of CML cases.[1] Bcr-Abl-induced tumorigenesis is a complicated process, involving the alteration of multiple signaling pathways that regulate cell survival and proliferation, including phosphatidylinositide 3-kinases/phosphatase and tensin homolog (PTEN)/AKT, RAS and Janus kinases/signal transducer and activator of transcription (STAT).[2–4] Although progress has been made in the understanding of signal transduction in the Abl transformation, the role of lncRNAs in these processes remains unknown.

Human genome is pervasively transcribed, and the majority does not have apparent protein-coding potential, which is defined as noncoding RNA (ncRNA).[5–7] LncRNAs are >200 nucleotides in length, transcribed by RNA polymerase II or III and poorly conserved.[8] Numerous functional lncRNAs have been identified and recognized as critical regulators of various physiological processes via controlling multiple levels of the gene expression.[9,10] LncRNAs function by virtue of the motifs embedded in their sequences that enable the specific association between lncRNAs and DNA, RNA or protein.[11,12] Importantly, aberrant expression of lncRNAs has been referred to as the hallmark of diverse human cancers.[13–15] For instance, the lncRNA, HOX antisense intergenic RNA, is greatly upregulated in both primary and metastatic breast tumors, which is correlated with high cancer metastasis and poor prognosis.[16,17] In addition, elevated expression of metastasis-associated lung adenocarcinoma transcript 1 was originally identified as a powerful predictor of high metastatic potential and poor prognosis in non-small-cell lung adenocarcinoma patients.[18] It has now been observed in a variety of human tumors, including those derived from breast, prostate, colon, liver and uterus.[19–21] Recently, lncRNA-LET has also been implicated in various tumor formations and may function as a tumor suppressor.[14] Interestingly, a number of lncRNAs, such as T-UCRs (transcribed ultraconserved regions), HOTTIP (HOXA transcript at the distal tip), ANRIL (antisense noncoding RNA in the INK4 locus), lincRNA-p21 (long intergenic noncoding RNA-p21) and MEG3 (maternally expressed 3), have been found to be associated with leukemia.[22,23] However, the molecular mechanisms underlying the involvement of lncRNAs in leukemia development are poorly understood.

Recently, a competitive endogenous RNA (ceRNA) hypothesis has been proposed.[24] In this hypothesis, RNAs can crosstalk with each other by competing for shared miRNAs. The tumor-suppressor PTEN has been validated to compete with several

[1]CAS Key Laboratory of Pathogenic Microbiology and Immunology, Institute of Microbiology, Chinese Academy of Sciences (CAS), Beijing, China; [2]School of Life Sciences, Anhui University, Hefei, China; [3]College of Animal Sciences, Fujian Agriculture and Forestry University, Fuzhou, China; [4]Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences and Comparative Medical Center, Peking Union Medical College, Beijing, China; [5]Center of Oncology and Hematology, The First Affiliated Hospital of Guangzhou Medical University, Guangzhou, China; [6]Institute of Biophysics, Chinese Academy of Sciences, Beijing, China and [7]Department of Biochemistry and Molecular Biology, Louisiana State University Health Sciences Center, Shreveport, LA, USA. Correspondence: Professor J-L Chen, CAS Key Laboratory of Pathogenic Microbiology and Immunology, Institute of Microbiology, Chinese Academy of Sciences (CAS), West Beichen Road, Chaoyang District, Beijing 100101, China.
E-mail: chenjl@im.ac.cn
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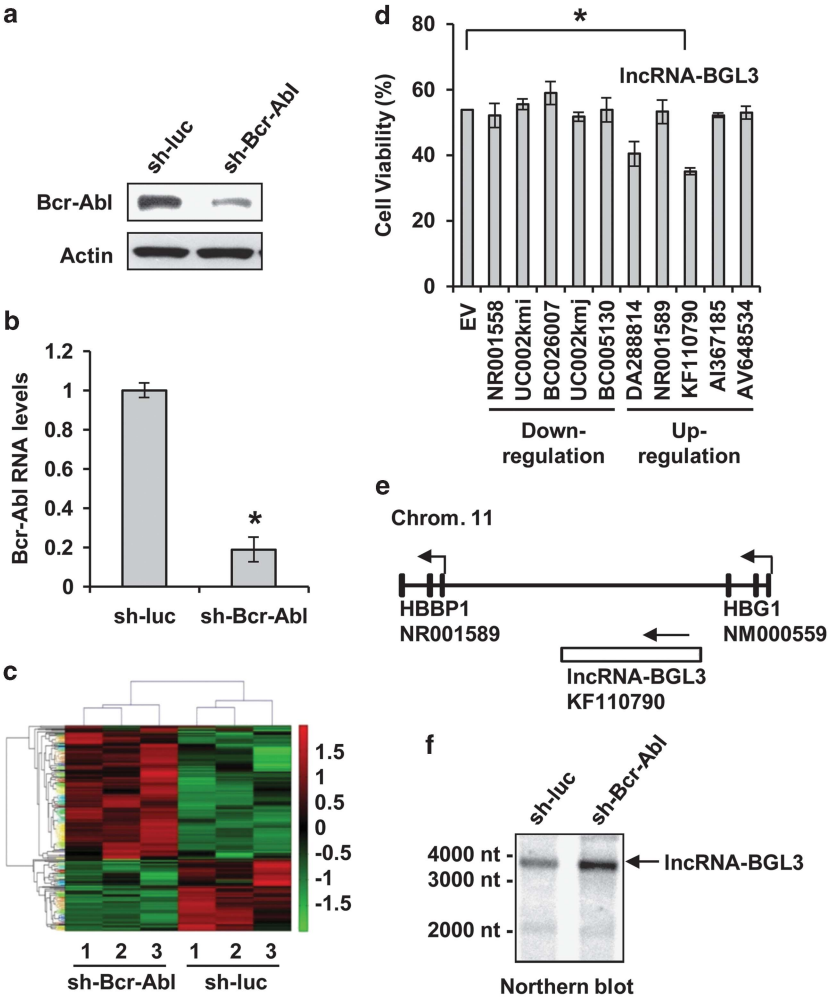

Figure 1. LncRNA-BGL3 is identified as a functional lncRNA whose expression is downregulated by Bcr-Abl. (**a**, **b**) Bcr-Abl expression in K562 cells expressing luciferase shRNA (sh-luc) or Bcr-Abl shRNA (sh-Bcr-Abl) was examined by western blotting (**a**) and quantitative real-time PCR (**b**). Results in panel (**b**) are presented as mean ± s.e.m. ($n = 3$), $^*P < 0.05$. (**c**) Shown is hierarchical clustering analysis of lncRNAs differentially expressed in K562 cells expressing luciferase shRNA or Bcr-Abl shRNA (greater than twofold differences; $P < 0.05$). The values from three independent experiments are displayed. The microarray analysis revealed 338 upregulated lncRNAs and 108 downregulated lncRNAs after silencing Bcr-Abl. (**d**) Cell survival analysis of K562 cells overexpressing selected lncRNAs or empty vector control (EV) treated with imatinib. Cell viability was assayed by flow cytometry after propidium iodide staining. Plotted are results from three independent experiments. Error bars represent s.e.m., $n = 3$, $^*P < 0.05$. (**e**) A schematic representation of the location of lncRNA-BGL3 between the hemoglobin gamma A (*HBG1*) gene and the hemoglobin beta pseudogene 1 (*HBBP1*) is shown. The exons and introns of HBG1 and HBBP1 genes are indicated by black boxes and black lines, respectively, and lncRNA-BGL3 is depicted as the grey box. The orientation of arrows indicates the direction of the transcription. (**f**) Northern blotting analysis of lncRNA-BGL3 expression in K562 cells. Equal amount of RNAs isolated from K562 cells expressing luciferase shRNA or Bcr-Abl shRNA were examined with ^{32}P-labeled lncRNA-BGL3-specific probe.

ceRNAs in diverse cancers. For example, VAPA (vesicle-associated membrane protein-associated protein A) and CNOT6L (CCR4-NOT transcription complex, subunit 6-like) were identified as ceRNAs for PTEN and displayed tumor-suppressive properties in prostate cancer.[25] ZEB2 (zinc finger E-box-binding homeobox 2) has been revealed to act as a decoy of miRNAs targeting PTEN and thereby regulates PTEN levels in melanoma.[26] Interestingly, a recent study has demonstrated that Linc-MD1 (long intergenic noncoding RNA-muscle differentiation 1) could control muscle differentiation by acting as a ceRNA. Linc-MD1 may sequester miR-133 and miR-135 to regulate their targets MAML1 (mastermind-like protein 1) and MEF2C (myocyte-specific enhancer factor 2C), transcription factors associated with the activation of muscle-specific genes.[27] PTENP1 (PTEN pseudogene 1) was found to affect PTEN level by functioning as a decoy of PTEN-targeting miRNAs.[28] These observations suggest that the ceRNA-based regulatory circuitry may represent a general phenomenon in physiological and pathological processes.[29]

In this study, we found that numerous lncRNAs differentially expressed in K562 leukemic cells in response to silencing of Bcr-Abl. One of these, lncRNA-BGL3 (NCBI accession number KF110790, AY034471) was greatly upregulated by disruption of Bcr-Abl expression or inactivation of Abl kinase in CML cell line and primary CML samples. LncRNA-BGL3 acted as a tumor suppressor during Bcr-Abl-induced tumorigenesis. There existed a miRNA-dependent reciprocal regulation mode between lncRNA-BGL3 and PTEN. These observations demonstrate the functional involvement of lncRNA-BGL3 in Bcr-Abl-mediated leukemogenesis and provide novel insights into complicated mechanisms underlying Bcr-Abl-induced hematopoietic malignancies.

RESULTS

Human lncRNA-BGL3 is identified as a functional lncRNA whose expression is downregulated by Bcr-Abl

In an attempt to provide insights into the mechanisms by which Bcr-Abl causes leukemogenesis and to identify lncRNAs involved in Bcr-Abl-mediated cellular transformation, microarray of cDNAs encoding lncRNAs was used to analyze differentially expressed lncRNAs in human K562 leukemic cells stably expressing specific short hairpin RNAs (shRNAs) targeting Bcr-Abl or luciferase control. The interference efficiency of these shRNAs in cells was determined (Figures 1a and b). Following disruption of Bcr-Abl expression, all the lncRNAs whose expressions were altered by at least twofold were clustered and displayed (Figure 1c). The microarray analysis revealed 338 upregulated lncRNAs and 108 downregulated lncRNAs after silencing Bcr-Abl.

To identify functional lncRNAs that are important in Bcr-Abl-mediated transformation, we selected the most significantly affected lncRNAs for further studies. These lncRNAs were examined by open reading frame finder from NCBI and no protein-coding potential was found. Furthermore, their expression was confirmed in Bcr-Abl knockdown K562 cells (Supplementary Figure S1a). Additionally, imatinib, an Abl tyrosine kinase inhibitor, was used to determine the effect of Bcr-Abl kinase inactivation on their expression. Interestingly, similar expression patterns were observed in the cells treated with imatinib (Supplementary Figure S1a). Next, the functional involvement of these lncRNAs in Bcr-Abl-mediated transformation was investigated. We generated K562 cell lines stably overexpressing the selected lncRNAs, respectively, and analyzed effects of these lncRNAs on cell survival. K562 cells underwent apoptosis following imatinib treatment, and approximately 54% of the control cells remained

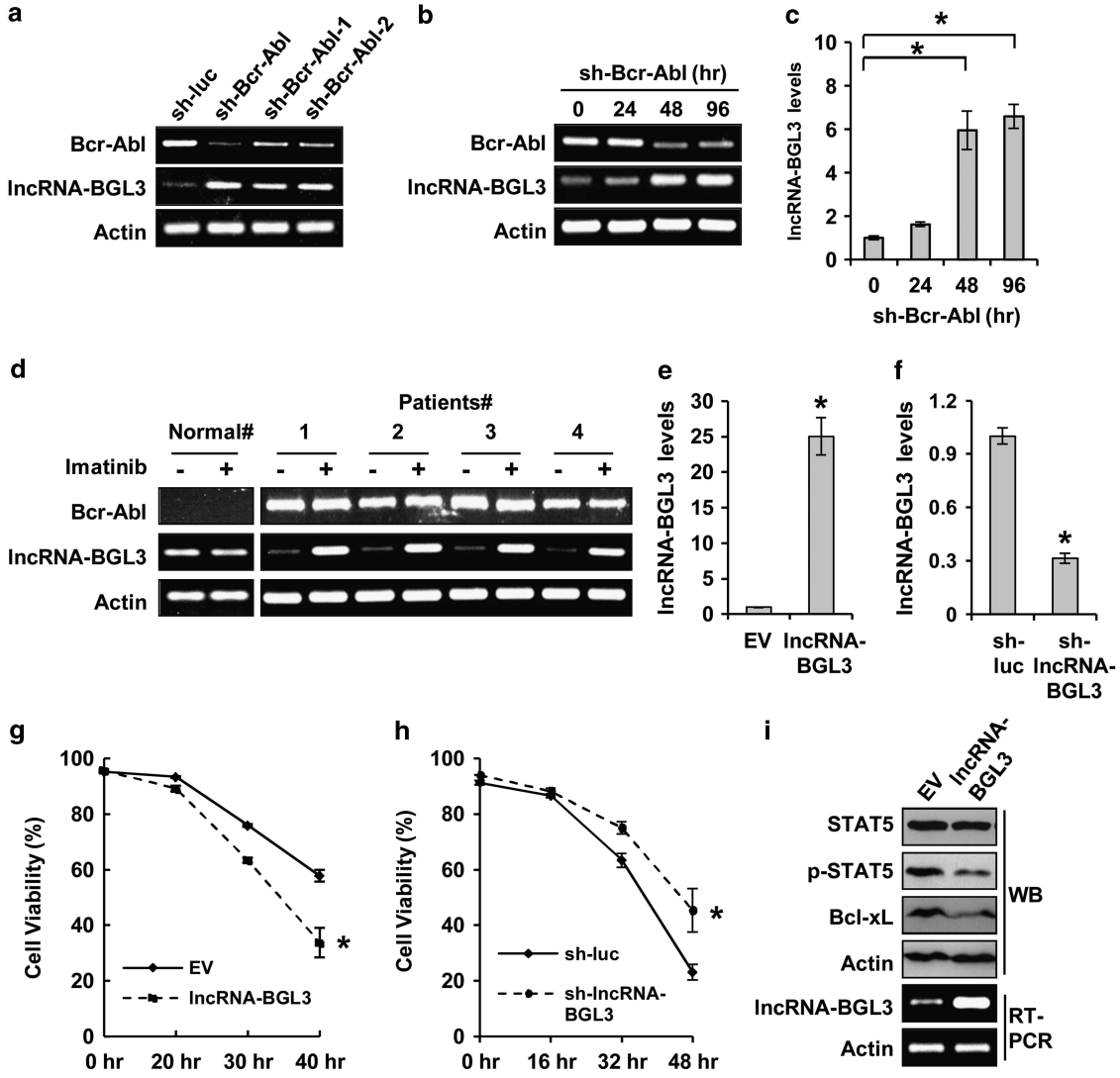

Figure 2. LncRNA-BGL3 sensitizes K562 cells to undergo apoptosis. (**a**) RT–PCR analysis of lncRNA-BGL3 expression in K562 cells expressing different Bcr-Abl-specific shRNAs that all target Bcr-Abl fusion point (also see Supplementary Figure S2a). (**b**, **c**) RT–PCR and quantitative real-time PCR analysis of lncRNA-BGL3 expression in K562 cells infected with lentivirus encoding sh-Bcr-Abl for the indicated time. Results in panel (**c**) are presented as mean ± s.e.m. ($n = 3$), $^*P < 0.05$. (**d**) RT–PCR analysis of lncRNA-BGL3 expression in primary CML cells from patients after exposure to imatinib (10 μM) for 24 h. (**e**, **f**) Quantitative real-time PCR was performed to examine the expression of lncRNA-BGL3 in stable K562 cells ectopically overexpressing lncRNA-BGL3 (**e**) or expressing lncRNA-BGL3 shRNA (**f**). Results are presented as mean ± s.e.m. ($n = 3$), $^*P < 0.05$. (**g**, **h**) Survival of K562 cells stably expressing lncRNA-BGL3, lncRNA-BGL3-specific shRNA or controls was analyzed by flow cytometry after treatment with imatinib. Results are presented as mean ± s.e.m. ($n = 3$), $^*P < 0.05$. (**i**) Western blotting and RT–PCR were performed to examine the protein and mRNA levels as indicated.

viable after incubation with this inhibitor for 40 h under our culture condition. In contrast, only approximately 35% of lncRNA-BGL3-expressing cells were viable under the same condition (Figure 1d). Importantly, overexpression of lncRNA-BGL3 had the most significant effect on K562 cell survival. Thus, it was pursued for studies in detail.

Previous studies indicated that BGL3 (Beta Globin Locus 3) was a noncoding RNA that may have a role in the regulation of γ-globin expression during development. BGL3 participates in γ-globin long-range contact with the locus control region and transcription activation.[30,31] However, precise function of BGL3 remains largely unknown. Previous data showed that BGL3 was a transcript of 993 nt (GenBank AY034471). We found that BGL3 was included in NONCODE v3.0, a publicly comprehensive and systematic ncRNA database,[32] and noted that it is located between the hemoglobin gamma A (*HBG1*) gene and the hemoglobin beta pseudogene 1 (*HBBP1*) on chromosome 11; 5265784–5269453 (Figure 1e). Consistent with the prediction based on *in silico* analysis of genomic sequences, northern blotting analysis demonstrated that BGL3 was a transcript of approximately 3670 nt and was markedly upregulated by silencing the Bcr-Abl in K562 cells (Figure 1f). Thus, this novel transcript was cloned by reverse transcriptase–PCR (RT–PCR), sequenced, renamed as lncRNA-BGL3 and submitted to GenBank database (GenBank KF110790). The open reading frames in the sequence were examined by open reading frame finder from NCBI[33] and demonstrated that lncRNA-BGL3 had poor Kozak strength and no protein-coding potential (Supplementary Figure S1b). We also determined the coding potential calculator score of lncRNA-BGL3 and performed a codon substitution frequency analysis using PhyloCSF.[34,35] LncRNA-BGL3 showed very low coding potential calculator and codon substitution frequency scores (−1.13569 and −2189.6095, respectively), indicating that it is a non-coding RNA.

LncRNA-BGL3 sensitizes Bcr-Abl-positive K562 leukemic cells to undergo apoptosis induced by imatinib

To further confirm that expression of lncRNA-BGL3 is regulated by Bcr-Abl, K562 cells were infected with different lentiviral shRNAs targeting Bcr-Abl or infected in a time course with the lentivirus. We found that silencing Bcr-Abl expression markedly increased the expression of lncRNA-BGL3 in K562 cells (Figures 2a–c, Supplementary Figure S2a). Similarly, treatment of the cells with imatinib led to increased expression of lncRNA-BGL3 (Supplementary Figures S2b–e). Moreover, four Bcr-Abl-positive CML samples derived from patients in chronic phases at diagnosis were utilized to determine the expression of lncRNA-BGL3. Strikingly, we observed that imatinib treatment greatly induced the expression of lncRNA-BGL3 in the leukemic cells from the CML patients but had no effect on normal control cells (Figure 2d).

Because expression of lncRNA-BGL3 is downregulated by Bcr-Abl kinase in both the leukemic cell line and primary CML cells, we hypothesized that lncRNA-BGL3 might have a key role in the oncogenic transformation. To test this possibility, we conducted gain-of-function studies by generating stable K562 cell lines overexpressing lncRNA-BGL3 and designed shRNA targeting lncRNA-BGL3 to disrupt its expression in the cells (Figures 2e and f; Supplementary Figures S2f and g). Subsequently, the effect of altered lncRNA-BGL3 expression on survival of K562 cells was investigated. We observed that 57.74% of control K562 cells remained viable after treatment with imatinib for 40 h under our culture condition. However, only 33.73% of the cells over-expressing lncRNA-BGL3 were viable under the same condition (Figure 2g), although there were only slight decreases in the survival and proliferation of cells expressing lncRNA-BGL3 in the absence of imatinib (Supplementary Figures S2h and i). These data indicate that lncRNA-BGL3 sensitizes K562 cells to undergo apoptosis in response to the drug treatment. Experiments testing the effect of silencing lncRNA-BGL3 on cell survival also demonstrated that depletion of lncRNA-BGL3 significantly promoted survival of K562 cells in the presence of imatinib (Figure 2h). Similarly, lncRNA-BGL3 overexpression also sensitized v-Abl-transformed cells to undergo imatinib-induced apoptosis (Supplementary Figures S2j–l). These results suggest that lncRNA-BGL3 is critical for imatinib-induced apoptosis in Abl-transformed cells.

In an attempt to provide insights into the mechanism by which lncRNA-BGL3 regulates survival of Bcr-Abl-expressing cells, we evaluated the activation of STAT5 and expression of Bcl-xL that are critical regulators of apoptosis in the leukemic cells.[36] The results showed that overexpression of lncRNA-BGL3 significantly inhibited STAT5 phosphorylation and decreased the levels of Bcl-xL protein in the cells (Figure 2i).

Altering lncRNA-BGL3 expression significantly affects tumor formation induced by K562 leukemic cells in xenograft mouse model

Next, we asked whether Bcr-Abl-mediated repression of lncRNA-BGL3 expression is required for Bcr-Abl-induced tumorigensis *in vivo*. To this end, we injected nude mice subcutaneously with K562 cells stably overexpressing lncRNA-BGL3 or empty vector control. Tumor growth was examined every week after inoculation. Strikingly, tumors formed by control cells grew much faster than those formed by cells expressing lncRNA-BGL3, whereas no tumors were induced by Bcr-Abl-silencing K562 cells (Figure 3a; Supplementary Figures S3a–c). By statistical analysis, we found that tumor growth was significantly impeded by ectopic expression of lncRNA-BGL3 (Figures 3b and c). At least three independent experiments were performed to ensure the specificity and consistency of these results. These observations were further confirmed by bioluminescent imaging analysis (Figure 3d), and overexpression of lncRNA-BGL3 in the tumors was determined by quantitative real-time PCR (Figure 3e). In addition, nude mice were injected subcutaneously with K562 cells stably expressing shRNAs targeting lncRNA-BGL3 or luciferase control. As expected, depletion of lncRNA-BGL3 promoted K562 xenografted tumor growth in nude mice (Figures 3f–h).

To further confirm the effect of lncRNA-BGL3 expression on Bcr-Abl-mediated tumorigensis, we used another vector, the lentiviral vector pll3.7, to ectopically express lncRNA-BGL3 in K562 cells. Similarly, lncRNA-BGL3 was shown to significantly promote the cells to undergo imatinib-induced apoptosis and inhibit Bcr-Abl-induced tumor growth *in vivo* (Figures 3i–k; Supplementary Figures S3d–g). These results demonstrated an inhibitory role of lncRNA-BGL3 in Bcr-Abl-induced tumorigenesis.

Expression of lncRNA-BGL3 in transgenic (TG) mice significantly inhibits Bcr-Abl-mediated primary bone marrow transformation

To further define the inhibitory role of lncRNA-BGL3 in Bcr-Abl-mediated cellular transformation, we were interested in establishing a more physiological model system to analyze the involvement of lncRNA-BGL3 in malignant transformation by Bcr-Abl oncogene. For this, we generated lncRNA-BGL3 expressing TG mice as previously described.[4,37] The TG founders with high expression efficiency were selected (Figure 4a; Supplementary Figure S4). Primary bone marrow cells derived from lncRNA-BGL3-expressing TG mice or their wild-type (WT) littermates were infected with the retrovirus encoding Bcr-Abl, and the ability of the virus to transform bone marrow cells was measured by counting the number of Bcr-Abl-transformed cell clones as previously described.[4,38] As shown in Figure 4b, WT cells infected with the retrovirus displayed Bcr-Abl transformation, with an average of 12.5 wells showing growth of transformed cell clones per 96-well plate. However, under the same condition, Bcr-Abl

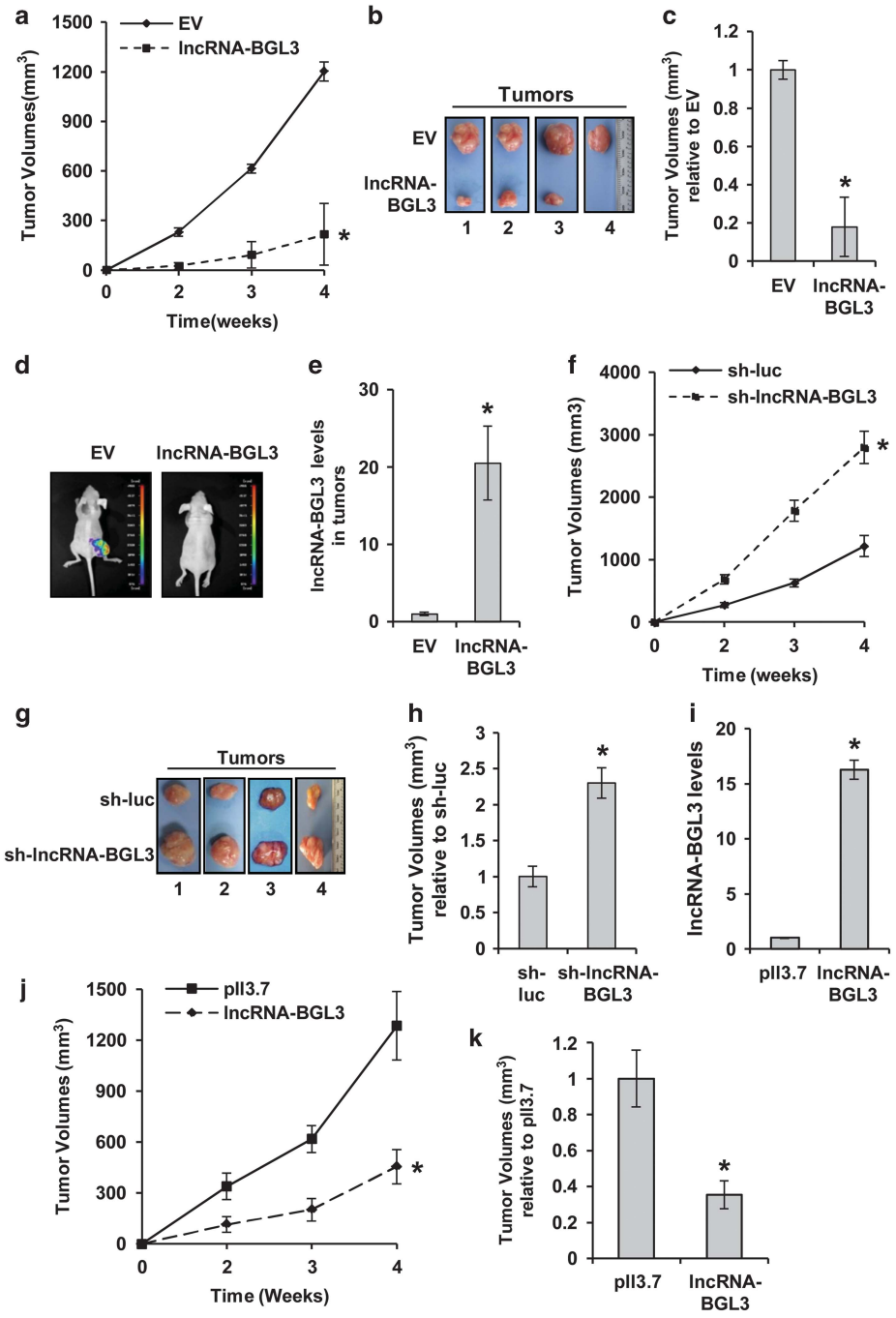

Figure 3. Altering lncRNA-BGL3 expression significantly affects tumor formation induced by K562 leukemic cells in xenograft mouse model. (**a**) Nude mice were subcutaneously injected with K562 cells overexpressing lncRNA-BGL3 (lncRNA-BGL3) or empty vector (EV). The tumor volumes were measured at the indicated time points. Plotted are results from three independent experiments. Error bars represent s.e. $n = 10$, $^*P < 0.05$. (**b**) Tumors were excised from mice. Shown are representative images from four independent experiments with similar results. (**c**) Relative volume of tumors excised from nude mice injected with K562 cells overexpressing lncRNA-BGL3 or control. The average volume of tumors induced by control cells is 1. Error bars represent s.e. $n = 10$, $^*P < 0.05$. (**d**) Over a 28-day period after inoculation, tumors caused by lncRNA-BGL3 overexpressing or control K562 cells were measured by bioluminescent imaging. Shown are representative images from at least three independent experiments with similar results. (**e**) LncRNA-BGL3 expression in representative tumors formed by K562 cells overexpressing lncRNA-BGL3 or control was examined by quantitative real-time PCR. Results are presented as mean ± s.e.m. ($n = 3$), $^*P < 0.05$. (**f**) Nude mice were subcutaneously injected with K562 cells expressing luciferase control shRNA (sh-luc) or lncRNA-BGL3 shRNA (sh-lncRNA-BGL3). The tumor volumes were measured at the indicated time points. Plotted are results from three independent experiments. Error bars represent s.e. $n = 10$, $^*P < 0.05$. (**g**) Shown are representative tumor images from four independent experiments with similar results. (**h**) Relative volume of tumors formed by lncRNA-BGL3 knockdown or control K562 cells. The average volume of tumors induced by control cells is 1. Error bars represent s.e. $n = 10$, $^*P < 0.05$. (**i**) Quantitative real-time PCR analysis of lncRNA-BGL3 expression in K562 cells expressing lncRNA-BGL3 or empty vector (pll3.7). Results are presented as mean ± s.e.m. ($n = 3$), $^*P < 0.05$. (**j**) Nude mice were subcutaneously injected with K562 cells overexpressing lncRNA-BGL3 or pll3.7. The tumor volumes were measured at the indicated time points. Plotted are results from three independent experiments. Error bars represent s.e. $n = 10$, $^*P < 0.05$. (**k**) Shown is relative volume of tumors caused by K562 cells overexpressing lncRNA-BGL3 or pll3.7. The average volume of tumors induced by pll3.7 cells is 1. Error bars represent s.e. $n = 10$, $^*P < 0.05$.

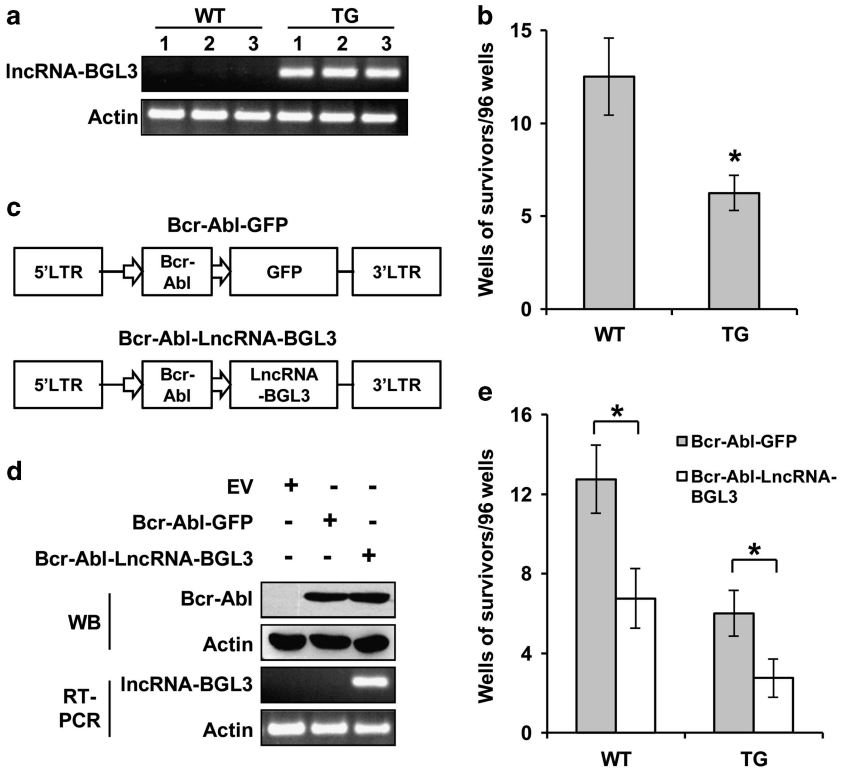

Figure 4. Expression of lncRNA-BGL3 in TG mice significantly inhibits Bcr-Abl-mediated primary bone marrow (BM) transformation (**a**) LncRNA-BGL3 expression in representative tissues (BM) from lncRNA-BGL3-expressing TG mice (TG) and WT littermates was examined by RT–PCR. (**b**) BM cells from WT and TG mice were infected with retroviruses expressing Bcr-Abl. Transformation efficiency was scored as described in Materials and Methods. Results are presented as mean ± s.e.m. ($n = 3$), $^*P < 0.05$. (**c**, **d**) Lentiviral vectors expressing Bcr-Abl and either GFP or lncRNA-BGL3 were constructed (**c**) and expression of Bcr-Abl and lncRNA-BGL3 was examined by western blotting and RT–PCR (**d**). (**e**) BM cells from WT and TG mice were infected with the lentiviruses expressing Bcr-Abl and either GFP or lncRNA-BGL3. Plotted are results from three independent experiments. Error bars represent s.e.m., $n = 3$, $^*P < 0.05$.

transformation efficiency significantly decreased to 6.25 wells per 96-well plate when bone marrow cells derived from TG were infected with equal titer of the virus.

To confirm this observation, we generated lentiviral constructs expressing Bcr-Abl and either GFP (green fluorescent protein) or lncRNA-BGL3 (Figures 4c and d). Indeed, we found that over-expression of lncRNA-BGL3 significantly reduced Bcr-Abl-mediated cellular transformation efficiency when bone marrow cells derived from WT mice were infected with the lentiviruses (Figure 4e). Similar results were obtained from experiments using the TG mice. Taken together, these results suggest that human lncRNA-BGL3 is functional in mice, and it has profound inhibitory effect on Bcr-Abl-mediated primary bone marrow transformation.

LncRNA-BGL3 is a target of miR-17, miR-20a, miR-20b, miR-93, miR-106a and miR-106b, microRNAs that repress messenger RNA of PTEN

To understand the mechanism by which lncRNA-BGL3 suppresses Bcr-Abl-mediated transformation, we used computational and experimental methods to identify the functional sites in lncRNA-BGL3 sequences as previously described.[39] Surprisingly, bioinformatics prediction using FINDTAR3 (http://bio.sz.tsinghua.edu.cn/) for miRNA recognition sequences in lncRNA-BGL3 indicated the presence of a putative binding site for a set of miRNAs, including miR-17, miR-20a, miR-20b, miR-93, miR-106a and miR-106b, which share the same seed sequence (CGUGAAA) (Figure 5a). We determined the expression levels of these miRNAs in K562 cells. Consistent with previous studies, we observed that they were all expressed in K562 leukemic cells (Supplementary Figure S5a).[40] To validate that the predicted microRNA target site is functional, we generated luciferase reporter constructs (Figure 5b). WT lncRNA-BGL3 or lncRNA-BGL3 mutant devoid of the specific miRNA-binding site was cloned downstream of the Renilla luciferase gene and transfected into 293T cells together with specific miRNAs mimics or the negative control mimic. Our results showed that luciferase expression was significantly reduced in cells transfected with lncRNA-BGL3 and specific miRNA mimics as compared with that in cells transfected with lncRNA-BGL3 and negative control. However, luciferase expression in cells transfected with lncRNA-BGL3 mutant and the miRNA mimics was comparable to that of control cells (Figure 5c). These data suggest that lncRNA-BGL3 is a target of miR-17, miR-93, miR-20a, miR-20b, miR-106a and miR-106b.

Importantly, these miRNAs have been shown to repress the mRNA of PTEN.[28] Thus, we compared the miRNA-dependent regulation of lncRNA-BGL3 with that of PTEN. Notably, highly similar miRNA-dependent regulation pattern was observed in cells expressing these targets with the miRNA mimics (Figure 5d). Then, the localization of lncRNA-BGL3 in K562 and human peripheral blood mononuclear cells was examined.[41] Cell fractionation experiments showed that lncRNA-BGL3 was localized both in the cytoplasm and nucleus of these cells, and there existed significant number of lncRNA-BGL3 molecules in the cytoplasm (Figure 5e, Supplementary Figures S5b and S5c). We next performed RNA immunoprecipitation to detect lncRNA-BGL3 and PTEN bound to Ago2, an essential component of a functional RNA-induced silencing complex.[42] We observed lncRNA-BGL3 and PTEN enrichment in the precipitate (Figure 5f). These results suggest that lncRNA-BGL3 and PTEN are both directly targeted by the shared miRNAs.

LncRNA-BGL3 regulates PTEN levels by functioning as a ceRNA

Recently, it has been proposed that ceRNAs sequester miRNAs to regulate mRNA transcripts carrying common miRNA response elements (MREs).[25,26] Our results presented above revealed that lncRNA-BGL3 and PTEN shared the same MRE in their sequences and displayed similar miRNA-dependent regulation pattern in

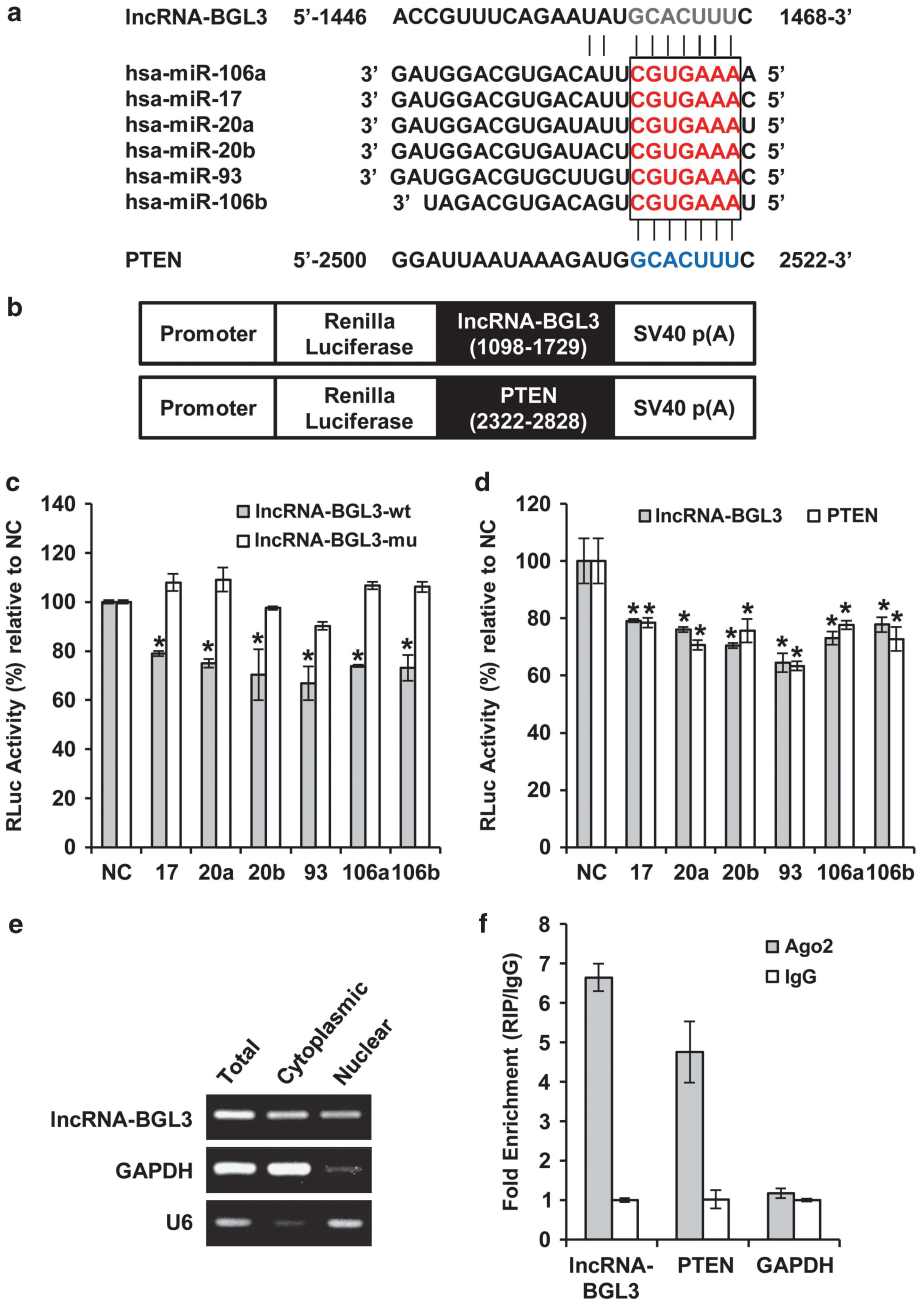

Figure 5. LncRNA-BGL3 is a target of miR-17, miR-93, miR-20a, miR-20b, miR-106a and miR-106b, microRNAs that repress mRNA of PTEN. (**a**) Nucleotide resolution of miRNA-binding sites in lncRNA-BGL3 and PTEN. As indicated, lncRNA-BGL3 contains a perfect seed sequence match with miR-17, miR-93, miR-20a, miR-20b, miR-106a and miR-106b that share the same seed sequence. These miRNAs are validated PTEN-targeting miRNAs. (**b**) Schematic representation of the constructs generated for luciferase assays. (**c**) LncRNA-BGL3 WT (lncRNA-BGL3-wt) or its mutant devoid of specific miRNA-binding sites (lncRNA-BGL3-mu) in which seed matches of lncRNA-BGL3/PTEN-targeting miRNAs were mutagenized from 'GCACTTT' to 'CCTCATA' was cloned downstream of Renilla luciferase gene (RLuc) in the vector pRL-TK and transfected into 293T cells together with specific miRNAs mimics or the negative control mimic (NC). Luciferase assay was performed as described in Materials and Methods. Plotted are results from three independent experiments. Error bars represent s.e.m., $n = 3$, $^*P < 0.05$. (**d**) LncRNA-BGL3 or PTEN-3′UTR was cloned downstream of Renilla luciferase gene, transfected into 293T cells together with specific miRNAs mimics or control and examined by the luciferase assay as described in panel (**c**). (**e**) K562 cells were fractionated into nuclear and cytoplasmic fractions. RT–PCR was performed to examine the levels of lncRNA-BGL3, nuclear control transcript (U6) and cytoplasmic control transcript (glyceraldehyde 3-phosphate dehydrogenase (GAPDH)) in these fractions. (**f**) Ago2 was immunoprecipitated (IP) from K562 cells, and co-precipitated RNAs were detected by quantitative real-time PCR using primers for lncRNA-BGL3, PTEN and GAPDH. IP enrichment is determined by the amount of RNA in precipitate associated with Ago2 relative to that in normal IgG control. Results are presented as mean ± s.e.m. ($n = 3$).

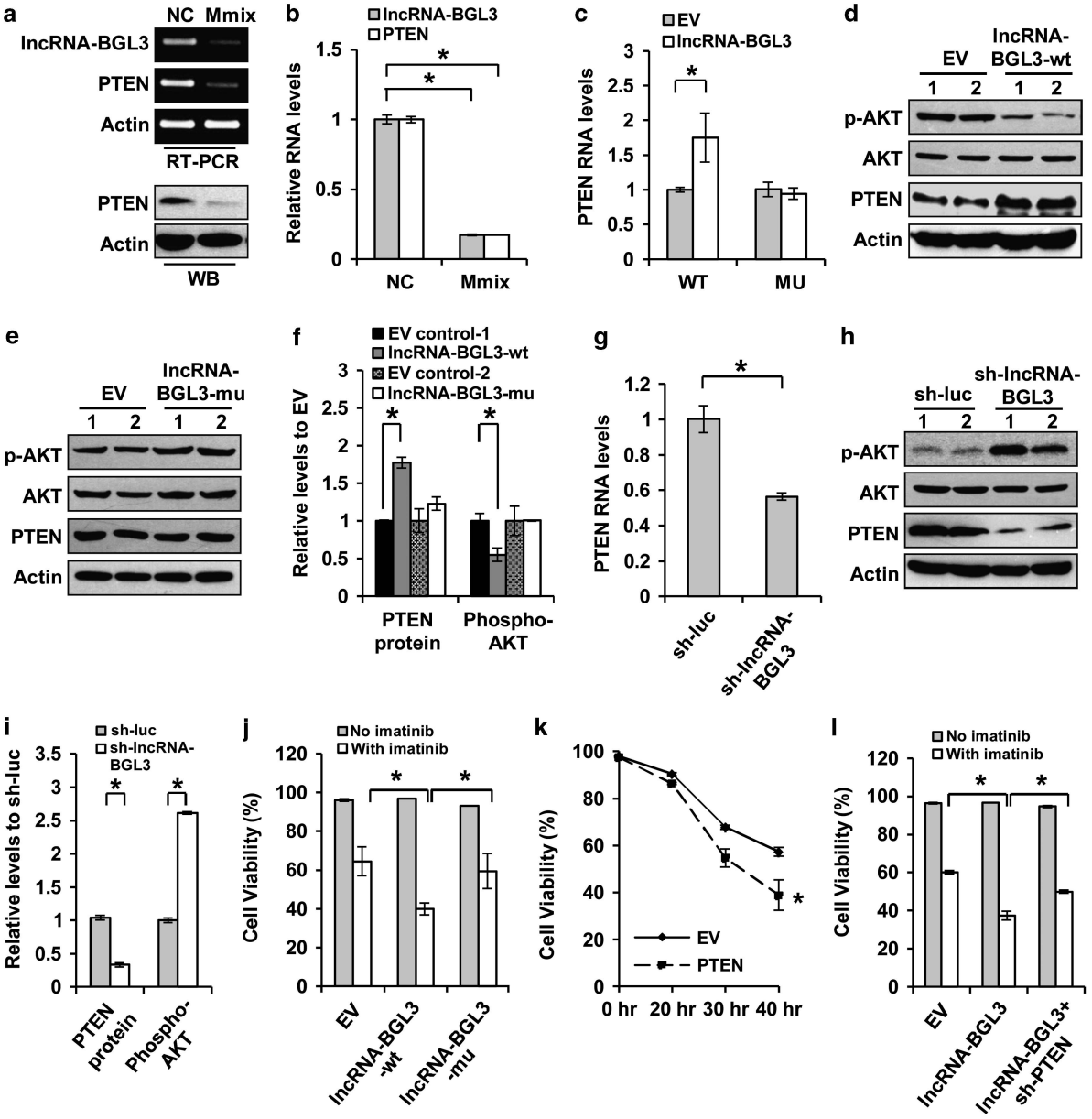

Figure 6. LncRNA-BGL3 acts as a ceRNA to regulate PTEN levels. (**a**, **b**) RT–PCR, quantitative real-time PCR and western blotting analysis of RNA and/or protein levels of lncRNA-BGL3 and PTEN in K562 cells transfected with specific miRNAs mimics (Mmix) or negative control mimic (NC). Results in panel (**b**) are presented as mean ± s.e.m. ($n = 3$), $^*P < 0.05$. (**c**) PTEN mRNA in K562 cells overexpressing lncRNA-BGL3 WT (lncRNA-BGL3-wt), its mutant devoid of the miRNA-binding sites (lncRNA-BGL3-mu) or empty vector (EV) was examined by quantitative real-time PCR. Results are presented as mean ± s.e.m. ($n = 3$), $^*P < 0.05$. (**d**, **e**) Shown are representative immunoblotting data from three independent experiments with similar results. Lysates from K562 cells expressing EV, lncRNA-BGL3-wt (**d**) or lncRNA-BGL3-mu (**e**) were probed with the indicated antibodies. (**f**) PTEN and phospho-AKT levels in panels (**d**) and (**e**) were quantitated by densitometry and normalized to actin and total AKT levels. The average value in EV control is 1. Plotted are the average levels from three independent experiments. The error bars represent the s.e.m. ($n = 3$), $^*P < 0.05$. (**g**) Quantitative real-time PCR analysis of PTEN transcript in control or lncRNA-BGL3 knockdown K562 cells. Results are presented as mean ± s.e.m. ($n = 3$), $^*P < 0.05$. (**h**) Western blotting analysis of PTEN and phospho-AKT levels in lncRNA-BGL3 knockdown or control K562 cells. (**i**) PTEN and phospho-AKT levels in panel (**h**) were quantitated as described in panel (**f**). (**j**) Cell viability of K562 cells overexpressing lncRNA-BGL3-wt, lncRNA-BGL3-mu or EV was assayed by flow cytometry after treatment with or without imatinib. Results are presented as mean ± s.e.m. ($n = 3$), $^*P < 0.05$. (**k**) Survival of PTEN overexpressing or control K562 cells was analyzed as described in panel (**j**). Results are presented as mean ± s.e.m. ($n = 3$), $^*P < 0.05$. (**l**) Survival of K562 cells overexpressing lncRNA-BGL3 alone, lncRNA-BGL3 and PTEN shRNA or EV was analyzed as described in panel (**j**).

luciferase reporter assay. These observations prompted us to examine whether there is a ceRNA-mediated regulation mode between lncRNA-BGL3 and PTEN. RT–PCR and western blotting were performed to determine endogenous levels of lncRNA-BGL3 and PTEN in K562 cells transfected with the mimics of these miRNAs. Indeed, we found that lncRNA-BGL3 exhibited concordant decreased expression pattern with PTEN in treated cells (Figures 6a and b). Furthermore, we generated stable K562 cell lines expressing PTEN 3′UTR (3′ untranslated region) or specific shRNA that targets PTEN. Noticeably, overexpression or

knockdown of PTEN correlated perfectly with the increased or decreased expression of lncRNA-BGL3, respectively (Supplementary Figures S5d and g).

Next, we investigated the ability of lncRNA-BGL3 to regulate the expression of endogenous PTEN in K562 cells. Our results showed that ectopic expression of WT lncRNA-BGL3 led to increased expression of PTEN, whereas expression of lncRNA-BGL3 mutant devoid of the MRE had no effect on PTEN level, indicating that the miRNA recognition sequences in lncRNA-BGL3 are required for lncRNA-BGL3-mediated regulation of PTEN expression (Figures 6c–f; Supplementary Figures S5h and i). By contrast, mRNA and protein levels of PTEN were markedly reduced in lncRNA-BGL3 knockdown cells (Figures 6g–i; Supplementary Figure S5j). Taken together, these data suggest that lncRNA-BGL3 and PTEN cross-regulate each other probably through sequestration of shared miRNAs.

Furthermore, we examined the functional involvement of the miRNA recognition sequences of lncRNA-BGL3 in leukemic cell survival. K562 cells expressing lncRNA-BGL3 and its mutant devoid of the MRE were analyzed for cell survival. Indeed, the lncRNA-BGL3 mutant exhibited impaired ability to affect K562 cell apoptosis (Figure 6j, Supplementary Figure S6a). Furthermore, several lncRNA-BGL3 truncated mutants were constructed based on the predicted secondary structure of lncRNA-BGL3 using RNAfold analysis (Supplementary Figure S6b). K562 cell lines stably overexpressing the mutants were generated and analyzed for cell survival (Supplementary Figures S6c and d). Interestingly, mutant (nt 1319–3670) containing the MRE had similar ability as lncRNA-BGL3 WT to sensitize K562 cells to undergo apoptosis, but remarkably, deletion of the region containing the MRE abolished this ability (Supplementary Figure S6e). These findings indicate that miRNA recognition sequences of lncRNA-BGL3 are essential for its function. However, expression of a smaller form (nt 1319–2713) containing the MRE was not sufficient to affect K562 cell survival (Supplementary Figures S6f and g), suggesting that expressing this part of lncRNA-BGL3 may alter its structure and thus affect its function. Additionally, our results showed that PTEN overexpression promoted imatinib-induced apoptosis of K562 cells, and depletion of PTEN significantly attenuated lncRNA-BGL3-mediated pro-apoptotic effect on K562 cells (Figures 6k and l; Supplementary Figures S6h and i), suggesting that lncRNA-BGL3 regulates cell survival, at least in part, through upregulation of PTEN levels in a miRNA-dependent manner.

Bcr-Abl represses lncRNA-BGL3 expression through c-Myc-dependent DNA methylation

Because disruption of Bcr-Abl expression or inhibition of Abl kinase led to a significant increase in lncRNA-BGL3 level, we asked next how lncRNA-BGL3 is downregulated by Bcr-Abl in CML cells. It is well recognized that epigenetic silence of tumor suppressors is one of key mechanisms that cause tumorigenesis, and upregulated expression of DNA methyltransferases DNMT1, 3A and 3B has been described in CML.[43] Thus, we explored the role of DNA methylation in regulating the expression of lncRNA-BGL3 that functioned as a tumor suppressor in Bcr-Abl transformation. To this end, we evaluated the effect of 5-Aza-2-deoxycytidine (5-Aza-dc), a specific DNA methyltransferase inhibitor, on lncRNA-BGL3 expression. Of interest, we observed that treatment with 5-Aza-dc significantly increased the level of lncRNA-BGL3 in K562 cells (Figures 7a and b). Furthermore, 5-Aza-dc treatment also resulted in upregulation of lncRNA-BGL3 expression in some human cancer cells, including acute T-cell leukemia cell line Jurkat and hepatocellular carcinoma cell lines 7402 and HepG2 but not in other cell lines, including 293T, hepatocellular carcinoma cell lines 7703 and Huh7, as well as breast adenocarcinoma MCF7 (Figure 7c; Supplementary Figure S7a), suggesting that expression and methylation of lncRNA-BGL3 may be cell line dependent.

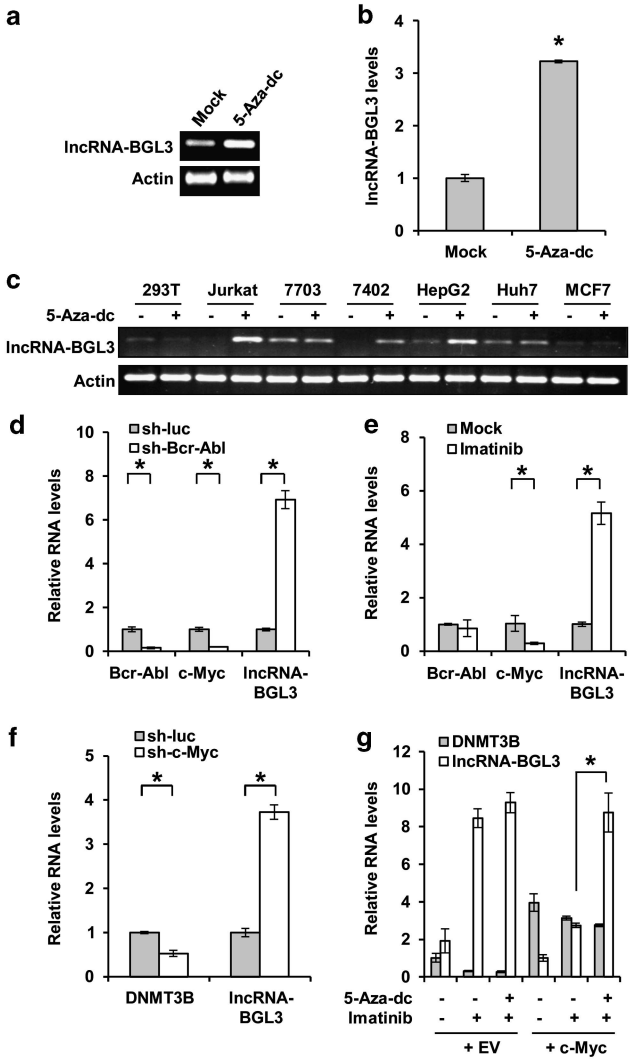

Figure 7. Bcr-Abl represses lncRNA-BGL3 expression through c-Myc-dependent DNA methylation. (**a**, **b**) RT–PCR and real-time PCR were performed to examine lncRNA-BGL3 expression in K562 cells treated with 5-Aza-dc (10 μM) for 72 h. Results are presented as mean ± s.e.m. ($n = 3$), $^*P < 0.05$. (**c**) RT–PCR analysis of lncRNA-BGL3 expression in 293T, Jurkat, 7703, 7402, HepG2, Huh7 and MCF7 cells treated with 5-Aza-dc. (**d**, **e**) Expression of c-Myc and lncRNA-BGL3 in Bcr-Abl knockdown or control K562 cells (**d**) or in cells treated with or without imatinib (**e**) was examined. Shown are data from real-time PCR analysis. Results are presented as mean ± s.e.m. ($n = 3$), $^*P < 0.05$. (**f**) The levels of lncRNA-BGL3 and DNMT3B in c-Myc knockdown or control K562 cells were examined by real-time PCR. Results are presented as mean ± s.e.m. ($n = 3$), $^*P < 0.05$. (**g**) Expression of lncRNA-BGL3 and DNMT3B in control or c-Myc-overexpressing K562 cells treated with imatinib alone, or imatinib and 5-Aza-dc, was examined by real-time PCR. Results are presented as mean ± s.e.m. ($n = 3$), $^*P < 0.05$.

It is well known that c-Myc is essential for transformation by Abl oncogenes, and c-Myc and Bcr-Abl cooperate to endow the transformed cells with cytokine-independent growth.[44,45] In addition, c-Myc contributes to enhanced expression of DNMT3A and 3B in different cancers.[46,47] Thus, we tested whether Bcr-Abl repressed lncRNA-BGL3 expression through c-Myc-dependent pathway. As expected, loss of Bcr-Abl expression or Abl kinase activity caused decline of c-Myc levels (Figures 7d and e; Supplementary Figures S7b and c). Importantly, silencing c-Myc expression alone in K562 cells remarkably increased the level of

lncRNA-BGL3 with simultaneously reduced level of DNMT3B (Figure 7f; Supplementary Figure S7d). In contrast, ectopic expression of c-Myc abrogated the imatinib-induced upregulation of lncRNA-BGL3 (Figure 7g; Supplementary Figure S7e). Notably, the addition of 5-Aza-dc could rescue the c-Myc-blocked lncRNA-BGL3 upregulation in the presence of imatinib (Figure 7g; Supplementary Figure S7e). Together, these data suggest that Bcr-Abl represses lncRNA-BGL3 expression probably through c-Myc-dependent DNA methylation.

DISCUSSION

It is well documented that malignant transformation of myeloid and lymphoid cells by Bcr-Abl involves the dysregulation or mutation of a variety of protein-coding genes that are normally involved in regulating the proliferation and survival of hematopoietic cells.[2,48,49] However, the functional relevance of lncRNAs and their *in vivo* regulation during Bcr-Abl-mediated tumorigenesis remain enigmatic. In this study, we identified that lncRNA-BGL3 was markedly upregulated by altering Bcr-Abl expression or its kinase activity in leukemic cells. We revealed that lncRNA-BGL3 overexpression sensitized leukemic cells to undergo apoptosis and inhibited Bcr-Abl-induced tumorigenesis *in vivo*. LncRNA-BGL3 TG mice exhibited impaired Bcr-Abl-mediated primary bone marrow transformation. These data provide strong evidence that lncRNA-BGL3 acts as a tumor suppressor during the Bcr-Abl-induced leukemogenesis. Importantly, upregulation of lncRNA-BGL3 by imatinib treatment was also detected in several primary CML patient samples, which express Bcr-Abl. We understand that our CML sample size is limited. Another large-scale study could increase the statistical power of our results obtained from CML samples. These remain an ongoing task.

Recently, growing evidence has demonstrated the critical roles of lncRNAs in various physiological and pathophysiological processes.[11,12,27] For example, lncRNAs may act as endogenous decoys for miRNAs to affect their distribution on specific targets. Here we show that lncRNA-BGL3 is a target of miR-17 family, which has also been found to repress mRNA of PTEN.[28] Because PTEN and its downstream AKT signaling have critical roles in Abl transformation,[50] we determined whether lncRNA-BGL3 might regulate Bcr-Abl-mediated transformation by competing for miRNAs targeting PTEN. Indeed, we found that altering the expression of lncRNA-BGL3 but not its mutant devoid of miRNA-binding sites had profound effect on the level of PTEN in K562 cells, indicating that the miRNA recognition sequences of lncRNA-BGL3 are required for lncRNA-BGL3-mediated regulation of PTEN. Interestingly, we observed that altering lncRNA-BGL3 expression had also effects on the levels of CNOT6L, VAPA and CDKN1A, three known targets of the miR-17 family (Supplementary Figure S8),[25,51] suggesting that lncRNA-BGL3 could affect whole networks of multiple targets. This miRNA-dependent reciprocal regulation mode shed a new light on the mechanisms underlying Bcr-Abl-induced tumorigenesis. On the other hand, our results suggest that lncRNA-BGL3 may modulate STAT5-dependent expression of anti-apoptotic Bcl-xL protein. A previous study has provided a direct link between the PTEN-AKT and STAT5 pathways.[52] However, further work is required to address the relationship of lncRNA-BGL3, PTEN-AKT and STAT5 pathway.

Recent studies revealed the existence of a previously unrecognized gene regulatory layer characterized by ceRNAs that compete for miRNA binding to cross-regulate the miRNA targets. CeRNAs-based gene regulation may form robust networks that when perturbed may lead to cancer.[29] Remarkably, it has been shown that regulation of the tumor-suppressor PTEN by ceRNA activity frequently occurs in several cancers, such as melanoma and prostate cancer.[25,26] The present report demonstrates for the first time that lncRNA-BGL3 functions as a ceRNA to suppress Bcr-Abl-positive leukemic growth through regulating PTEN and its downstream AKT signaling, and silencing its expression appears to downregulate PTEN level, tipping cells towards cancer progression. These results suggest a potential strategy for treatment of Bcr-Abl-induced malignancies.

Although BGL3 has been previously described,[30,31] little is known about its *in vivo* regulation. Here, we show that lncRNA-BGL3 is downregulated in the cells by DNA methylation. We observed that expression of c-Myc in K562 cells was Bcr-Abl dependent, and silencing c-Myc expression alone markedly increased the level of lncRNA-BGL3, with reduced levels of DNMT3B. Ectopic expression of c-Myc abrogated the imatinib-induced expression of lncRNA-BGL3, whereas 5-Aza-dc reversed the inhibitory effect of c-Myc on lncRNA-BGL3 upregulation by imatinib. These observations indicate that Bcr-Abl represses lncRNA-BGL3 expression likely through c-Myc-dependent DNA methylation. Together, the results reveal a mechanism of how Bcr-Abl may overcome lncRNA-BGL3 suppression to constitutively activate the AKT-dependent signaling. However, it is unclear whether regulation of lncRNA-BGL3 is important in physiological (non-CML) setting. There has been a report of an enhancer-like function for lncRNA in human cells.[53] Given the role of the BGL3 in regulating γ-globin transcription activation, it is interesting to explore the possible role of lncRNA-BGL3 as an enhancer lncRNA. Further studies are needed to address these issues and to determine the therapeutic significance of lncRNA-BGL3 in leukemia.

MATERIALS AND METHODS

Microarray and data analysis

The lncRNA cDNA microarray was from Arraystar (Arraystar, Rockville, MD, USA). Total RNAs from three independent groups of K562 cells expressing luciferase shRNA or Bcr-Abl shRNA were prepared using Trizol reagent (Invitrogen, Carlsbad, CA, USA). cDNA synthesis, labeling, hybridization and data analysis were carried out as previously described.[54] Our microarray data have been deposited in the NCBI Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/; access number GSE42269).

Cell lines and cell culture

Cell lines K562, 293T and Jurkat were purchased from American Type Culture Collection (Manassas, VA, USA). Hepatocellular carcinoma cell lines QGY-7703, BEL-7402, HepG2 and Huh7 and breast carcinoma cell MCF-7 were purchased from National Platform of Experimental Cell Resources for Sci-Tech (http://cellresource.cn, Beijing, China). Cells were grown in Dulbecco's modified Eagle medium or RPMI1640 supplemented with 10% fetal bovine serum and antibiotics (penicillin and streptomycin) as previously described.[4] LncRNA-BGL3 overexpressing K562 cells were generated by infecting the cells with viruses encoding lncRNA-BGL3 using the viral vector pMSCV-GFP or pll3.7 as previously described.[4] shRNA-expressing stable K562 cells were generated by infection of the cells with lentiviruses expressing specific shRNAs as described previously.[4] The shRNA sequences are described in Supplementary Materials and Methods.

Apoptosis assay

Cells were treated with imatinib (10 μM) for the indicated time, then stained with propidium iodode (PI)/Annexin V or PI and analyzed by fluorescence-activated cell sorter (BD Biosciences, San Jose, CA, USA).

Nude mice injection

Nude-mouse injection was performed as previously described.[4,38] Tumor growth was monitored and measured in volume (length × height × width) at the indicated time after inoculation. Bioluminescent imaging was used to probe tumor growth from GFP-expressing cells. Images were quantified as photons/s using the Indigo software (Berthold Technologies, Bad Wildbad, Germany).

Luciferase assay

LncRNA-BGL3 (lncRNA-BGL3-wt), its mutant devoid of specific miRNA-binding sites (lncRNA-BGL3-mu) or PTEN-3′UTR was cloned into 3′UTR of the Renilla luciferase gene in the vector pRL-TK (Promega, Madison, WI, USA). Each plasmid was transfected into cells, together with specific miRNAs mimics or with a negative control mimic (RiboBio, Guangzhou, China). Firefly luciferase gene in the vector pGL3-control (Promega) was used as a control for transfection efficiency. Luciferase assays were performed using the dual-luciferase reporter assay system kit (Promega) according to the manufacturer's instructions. Luciferase expression was analyzed by Modulus single-tube multimode reader (Promega). The relative luciferase expression equals the expression of Renilla luciferase (pRL-TK) divided by the expression of firefly luciferase.

Generation of lncRNA-BGL3-expressing TG mice

The lncRNA-BGL3-expressing TG mice were generated by the microinjection method as previously described.[4,37] RT–PCR was performed to determine the expression efficiency. The TG founders with high expression efficiency were selected and maintained on a C57BL/6J genetic background.

Primary murine bone marrow transformation assay

Bcr-Abl-mediated bone marrow transformation was carried out as previously described.[3,55] Transformation efficiency was scored by counting the number of the wells that displayed cytokine-independent growth 3 weeks postinfection.

Antibodies and reagents, DNA construction, cellular fractionation, RT–PCR, quantitative real-time PCR, western blotting, RNA immunoprecipitation and statistical analysis

Antibodies and reagents, DNA construction, cellular fractionation, RT–PCR, quantitative real-time PCR, western blotting, RNA immunoprecipitation and statistical analysis are described in the Supplementary Materials and Methods.

ABBREVIATIONS

lncRNA-BGL3, long noncoding RNA-Beta Globin Locus 3; PTEN, phosphatase and tensin homolog; miRNA, microRNA.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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